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Gingival Tissue Human Beta-Defensin Levels in Relation to Infection and Inflammation

Running Title: Human Beta-Defensins in Periodontitis

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Abstract

Aim: To profile gingival tissue levels of human beta-defensin (hBD)-2 and hBD-3 in relation to gingival inflammation, Th17-related cytokine concentrations, *Porphyromonas gingivalis* counts, and gingipain and total protease activities.

Materials and Methods: Gingival tissue and subgingival plaque samples were collected from 21 periodontitis patients including 48 periodontal pocket sites with marginal, mild, or moderate to severe inflammation. hBD levels were determined by immunodetection, *P. gingivalis* counts with real-time polymerase chain reaction, protease activities with fluorogenic substrates, and cytokine concentrations with Luminex technique. Data were statistically analyzed using Kruskal-Wallis and Mann-Whitney U tests and Spearman correlation coefficients.

Results: Subgingival plaque counts of *P. gingivalis* ($p=0.001$) and gingipain activity ($p<0.001$), as well as interleukin (IL)-1β ($p=0.012$), IL-10 ($p=0.024$), IL-17A ($p=0.002$), IL-17F ($p=0.006$), and IL-23 ($p=0.036$) concentrations were elevated in severely inflamed sites, whereas no change was observed in hBD-2 and hBD-3 levels. Negative correlations were found between protease activity and hBD-2 ($p=0.033$) and hBD-3($p=0.003$) levels.

Conclusions: Shift in gingival inflammation from marginal to mild stage is related to elevations in subgingival plaque *P. gingivalis* counts and gingipain activity, but not to tissue hBD levels. Negative correlations between hBDs and total protease activity suggest the degradation of these antimicrobial peptides in progressed inflammation.

Keywords: Periodontitis, antimicrobial peptides, hBD-2, hBD-3, *Porphyromonas gingivalis*, gingipain, inflammatory cytokines.

Clinical Relevance:

Scientific rationale for study: Human beta-defensins (hBDs) are small cationic peptides with antimicrobial and immune-regulatory roles and their expression and secretion are dependent on environmental (bacterial and inflammatory stimulation) and genetic factors.

Principal findings: Here, we demonstrated that the tissue hBD-2 and hBD-3 levels negatively correlate with enzymatic activity while Th17-related cytokine concentrations, *Porphyromonas gingivalis* counts, and gingipain activities relate to clinical gingival inflammation.

Practical implications: Understanding the activation and inactivation mechanisms of these antimicrobial and immune-regulatory peptides may enable to benefit from these biomolecules within the context of adjunctive periodontal therapy.

Introduction

Human beta-defensins (hBDs) are small, cysteine-rich cationic peptides with bi-directional regulatory relations to the adaptive immune system, angiogenesis, and wound healing (Suarez-Carmona et al., 2015). Gingival hBDs are produced by epithelial cells (Gomes and Fernandes, 2010) and are released into saliva (Gürsoy et al., 2016) and gingival crevicular fluid (Ertugrul et al., 2014, Yilmaz et al., 2018). While hBD-1 is secreted constitutively in periodontal tissues, hBD-2 and hBD-3 secretions are activated by bacterial or inflammatory stimuli (Ganz, 2003).

Porphyromonas gingivalis is a Gram-negative, anaerobic, asaccharolytic, non-motile bacterium (Darveau et al., 2012). *P. gingivalis* gingipains are cysteine proteinases with a trypsin-like activity, including lysine-specific gingipain (Kgp) and arginine-specific gingipains (RgpA and RgpB); they account for 85% of the total proteolytic activity of *P. gingivalis* (Potempa et al. 1997). Gingipains selectively subverts the host's innate and adaptive immunity by inactivating anti-inflammatory cytokines and degrading antimicrobial peptides, including hBDs (Carlisle et al., 2009; Maisetta et al. 2011).

On one hand, cytokines, such as interleukin (IL)-1β, IL-6, and IL-23, influence the differentiation of T helper 17 (Th17) cells. As a consequence, the increased release of Th17-related cytokines induces epithelial hBD expression (Kolls et al., 2008). On the other hand, infection either

stimulates or suppresses the secretion of these antimicrobial peptides, depending on the virulence of the bacteria involved (Diamond and Ryan, 2011, Gursoy et al., 2012). Here, we hypothesized that clinical inflammation and Th17-related inflammatory cytokines activate; whereas *P. gingivalis*, its gingipain activity, and total protease activity suppress hBD levels in periodontally inflamed gingival tissue. In the present study, the aim was to profile the levels of hBD-2 and hBD-3 in tissue samples with a specific reference for the severity and extent of clinical gingival inflammation and further, to correlate them with hBD-activating Th17-related cytokine concentrations, *P. gingivalis* counts, and hBD-degrading gingipain and total protease activities.

Materials and Methods

Ethical Guidelines

The study protocol (no: GO 17/786-34) was approved by the Ethical Committee of Hacettepe University, Ankara, Turkey in accordance with Helsinki Declaration 1975, as revised in year 2000. Oral and written information about the study protocol were given to potential participants. An informed consent was obtained from those willing to participate in the study.

Study Population

For the sample size calculation, GLIMMPSE 2.0.0 (General Linear Multivariate Model & Sample Size), which is a tool for calculating power and sample size in multilevel and longitudinal studies with an α=0.05 and β=0.10 targeting for 90% power with three response variables, was used. In the calculations, mean values and standard deviations for *P. gingivalis* α-haemolytic, β-haemolytic and non-haemolytic counts were based on a study by Wong et al. (Wong et al., 2016). Since changes in variability can dramatically affect power and sample size results, a scale factor was used to evaluate the sample size in case of variability in the present study is bigger than that in the reference study. It was assumed that the correlation coefficient between periodontal pockets with and without infection would be 0.3 and the correlation coefficient between the different responses would be 0.7. These analyses indicated that a required sample size would be a minimum of 16 participants with 48 sites.

Twenty-one periodontitis patients (13 males and 8 females with age range of 24-70 years) referred to the Hacettepe University, Faculty of Dentistry, Department of Periodontology, were recruited for the study. These periodontitis patients were the first to apply for the study and fit to the inclusion criteria [met the criteria of the 2017 World Workshop for classification of periodontal and peri-implant diseases (Papapanou et al., 2018)]. Exclusion criteria included systemic diseases, smoking, pregnancy, lactation, use of antibiotics and/or anti-inflammatory drugs within 3 months preceding the study and periodontal treatment within 6 months before the baseline.

Periodontal Examination

A full periodontal examination was performed by a single calibrated examiner (M.Ö.). Probing pocket depth (PPD), modified gingival index (MGI) (Lobene, 1986), clinical attachment level (CAL), plaque index (PI) (Löe, 1967) and bleeding on probing (BOP) (Ainamo and Bay, 1975) were recorded at 4 sites (mesio-buccal, mid-buccal, disto-buccal, lingual/palatinal) per tooth by using a manual periodontal probe (UNC-15, Hu-Friedy, Chicago, IL, USA). Orthopantomographs were taken (Sirona Orthophos XG5, NY, USA) from all individuals to evaluate the presence and extent of alveolar bone loss at the sampling sites. The extent of alveolar bone loss (ABL) was graded into three categories: mild: ABL in cervical third of the root; moderate, ABL in the middle third of the root; and severe, ABL from the apical third of the root (Salminen et al., 2014).

A total of 48 periodontal pocket sites (2 to 3 sites/patient) with PPD of 5-7 mm were included in the study. All pocket sites with PPD 5-7 mm were included unless the patient had more than one periodontal pocket with the same degree of inflammation and pocket depth. In such cases, only the tooth site that best reflects the degree of inflammation and being easily isolated (e.g. maxilla, anterior region) was enrolled for sample collection.

Based on the degree of inflammation as assessed by the MGI, the sampling sites were divided into three study groups:

Group 1: Marginal inflammation, slight changes in color and texture but not in all portions of gingival margin or papilla (n=13),

Group 2: Mild inflammation, slight changes in color and texture in all portions of gingival margin or papilla (n=16),

Group 3: Moderate to severe inflammation, bright surface, erythema, edema, ulceration or spontaneous bleeding tendency (n=19).

Levels of clinical parameters (PPD, MGI, CAL, PI, ABL) for each group are given in Table 1.

Clinical Sample Collection

Subgingival plaque was collected by paper points as described elsewhere (Belibasakis et al., 2014). Briefly, after removing the supragingival plaque, 3 sterile absorbent paper points (size 40, VDW, Munich, Germany) were gently inserted apically into the same periodontal pocket, removed after 15 s and placed in an Eppendorf tube containing 500 μl of phosphate-buffered saline (PBS).

Inflamed gingival granulation tissue was excised from the lateral pocket wall and junctional epithelium by using the sharp side of a Gracey curette (Hu-Friedy, Chicago, IL). Excision involved the entire pocket starting from the bottom of the periodontal pocket and ended at the marginal gingiva, preferably with a single stroke. Granulation tissue samples were placed in an Eppendorf tube containing 100 μl of PBS. The tissue samples and the paper points were immediately placed and stored at -80°C and transferred with dry ice to the Institute of Dentistry, University of Turku, Finland, for the laboratory analyses.

Sample Preparations

Each bacterial plaque sample was vortexed for 30 s and the suspension was aliquoted for analyses of gingipain and total protease activities and *P. gingivalis* concentrations.

Each granulation tissue sample was cut in small pieces, put into 500 μl of lysis buffer (50 mM Tris-Cl, 150 mM NaCl and 1% Triton X-100), vortexed for 10 s, and incubated at 4°C for 24 h. After incubation, tissues were centrifuged at 10 000 g for 1 min and supernatants were obtained. Activation of proteases during the extraction process was inhibited by performing the entire procedure at 4°C. The supernatants were aliquoted for determinations of gingipain and total protease activities, and *P. gingivalis*, hBD-2, hBD-3, and inflammatory cytokine concentrations.

Determination of *P. gingivalis* Counts

For the DNA extractions, 250 μl of bacterial plaque samples and 100 μl of gingival granulation tissue samples were transferred to the bead tubes (NucleoSpin® Microbial DNA kit, MacheryNagel) and mixed with 250 μl MG binding buffer and 25 μl proteinase K. Tubes were agitated in Mixer Mill MM301 (Retch) for 12 min at 30 Herz and centrifuged for 30 s at

11 000g. 150 µl of supernatant was transferred to a new tube and DNA was extracted with phenol-chloroform method and precipitated with 3 M NaAc (Green and Sambrook, 2017).Pelleted DNA was dissolved into 50 µl of TE-buffer.

P. gingivalis counts in gingival granulation tissue (including both tissue resident and epithelial adherent bacterial cells) and bacterial plaque samples were analyzed using a quantitative single copy gene-based real-time polymerase chain reaction (qPCR) as previously described (Hyvärinen et al., 2009) with some modifications. Reaction mixtures (total volume 20 µl) contained 2 µl of template DNA, 200 nM primers (Thermo Fisher Scientific) and 1x Universal KAPA SYBR FAST qPCR mastermix (KAPA Biosystems, Merck) supplemented with 1x ROX Low reference dye. Tenfold dilution series of the pJet1.2/blunt-Pg plasmid containing the whole *Pg waaA*-gene, encoding 3-deoxy-Dmanno-oct-2-ulosonic acid (Kdo) transferase, were used to generate standard curve. qPCR analyses were performed with the QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific) with following steps: Initial denaturation at 95°C for 3 min, followed by 40 cycles of 3s at 95°C and 20s at 60°C. Dissociation curve was generated according to the default settings of the QuantStudio 5 real time PCR system. DNA extracted from saliva samples of *P. gingivalis* -positive subjects were used as a positive control and water as a negative control in each set of analysis. The data were analyzed with QuantStudio™ Design and Analysis Software and the results are presented as genomic copies/ng DNA. The detection limit for *P. gingivalis* was 23 genome equivalents, calculated from the standard curves used.

Determination of Gingipain and Total Protease Activities

To determine the *P. gingivalis* specific gingipain activity, fluorogenic substrates that contain D-amino acids [*FITC-Ahx-(L)Arg-(D)Arg-KDbc* (RR)] and L-amino acids [(*FITC-Ahx-(L)Arg-(L)Arg-KDbc*) (rR)] were used (Kaman et al., 2012). The substrates were specifically developed for *P. gingivalis* and its gingipains, and their validation with positive controls has been previously published (Kaman et al., 2012; Galassi et al., 2012). A non-specific fluorogenic substrate, PEK-054, was used to analyze the total protease activity in the samples (Janus et al., 2015).

Either 16 µM of gingipain substrates or 8 µM of PEK-054 were added in 50 µl of plaque suspension, tissue supernatant or PBS as a negative control, all supplemented with 2.5 µM of L-cysteine. The 96-well plates were read at 37°C for 1 h with 2-min intervals on a fluorescence microplate reader with Gen5 software (Ex:485 nm – Em: 530 nm, Biotek Instruments, Winooski, VT, US). The protease activity was defined in relative fluorescence activity per min (RF/min) as

previously described (Kaman et al., 2012, Janus et al., 2015). All enzyme tests were performed in triplicate and repeated 3 times.

Determination of hBD-2 and hBD-3 by Immunodetection

Protein concentrations of each sample were determined by the Bradford method (Bio-Rad Laboratories Inc., Hercules, CA, USA). A same amount of protein (10 µg/mL, 144 ng) for each sample was mixed with 5 µl of Laemmli sample buffer (4x), and the mixture was heated at 95°C for 5 min. Synthetic hBD-2 (0.034 mM, 4338-s, Peptide Institute, Inc., Osaka, Japan) and hBD-3 (0.034 mM, 4382-s Peptide Institute Inc.) were used as controls. The samples and purified proteins were loaded on 15% sodium dodecyl sulfate (SDS)-polyacrylamide gels at non-reducing conditions. After electrophoresis, the proteins were transferred to 0.2 µm PVDF membranes (Trans-Blot® Turbo™ Transfer System, Bio-Rad Laboratories Inc.). Membranes were blocked with 5% non-fat dry milk at room temperature for 1 h and incubated overnight with primary antibodies against hBD-2 (1:1000 dilution, #AF2758, R&D Systems, Minneapolis, MN) and hBD-3 (1:750 dilution, # PA1-4216Thermo Fisher Scientific, Inc., Waltham, MA) at 4°C. To detect hBD-2, the membranes were incubated with HRP conjugated polyclonal rabbit anti-goat IgG (H+L) secondary antibody (1:2000 dilution #81-1620, 4216Thermo Fisher Scientific, Inc., Waltham, MA). To detect hBD-3, the membranes were incubated with HRP conjugated polyclonal goat anti-rabbit IgG secondary antibody (1:2000 dilution, LS-C60884, LSBio, Seattle, WA). Loaded protein levels were controlled with Coomassie blue staining (Welinder and Ekblad, 2011) and protein transfer was controlled with Ponceau S red stain. The proteins were visualized using Clarity™ Western ECL Substrate (0.6 ml/cm2 area, #170-5060, Bio-Rad Laboratories, Hercules, CA) and ChemiDoc™ MP Imaging System (Bio-Rad Laboratories). The bands were analyzed with the ImageJ software (National Institute of Health, Bethesda, Maryland, USA). All experiments were performed in triplicate at 2 different time points. Results of the Western-Blot analysis were additionally verified by using different primary antibodies than described above (for hBD-2 LSBio LS-B13646, Seattle, WA used with 1:1000 and 1:500 dilutions and for hBD-3 LSBio LS-C40649, Seattle, WA used with 1:500 dilution). Control experiments were performed by omitting the primary antibody from the immunodetections.

Cytokine Concentrations

Tissue concentrations of IL-1β, IL-4, IL-6, IL-10, IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-25, IL-31, IL-33, interferon gamma (IFN-γ), soluble CD40 ligand (sCD-40L), and tumor necrosis factor-

α (TNF-α) were detected by the Luminex technique (Bio-Rad, Santa Rosa, CA, USA) with the commercial kits (Bio-Plex, pro-human Th17 cytokine assays; Bio-Rad) according to the manufacturer's instructions. Tissue extracts were vortexed, centrifuged at 9300 *g* for 10 s, and a same amount of protein was taken from each tissue extract to determine the cytokine concentrations. The limit of detection of the assay was 0.02 pg/ml for IL-1β, 0.52 pg/ml for IL-4, 0.67 pg/ml for IL-6, 0.30 pg/ml for IL-10, 0.49 pg/ml for IL-17A, 0.80 pg/ml for IL-17F, 2.13 pg/ml for IL-21, 0.30 pg/ml for IL-22, 1.55 pg/ml for IL-23, 0.07 pg/ml for IL-25, 0.49 pg/ml for IL-31, 0.58 pg/ml for IL-33, 0.43 pg/ml for IFN-γ, 0.41 pg/ml for sCD-40L, and 0.07 pg/ml for TNF-α.

Statistical Analyses

Data distributions were analyzed with Q-Q plots and the Shapiro-Wilk test. The IBM SPSS V24.0 software (IBM, Armonk, North Castle, New York, USA) was used for statistical analyses, including the non-parametric Kruskal-Wallis (for multiple comparisons) and Mann–Whitney U tests and $p < 0.05$ was accepted as statistically significant. Associations between variables were evaluated with Spearman correlation coefficients.

Results

There were no significant differences in CAL, PPD, and ABL levels among the study groups. PI levels were significantly higher at Group 3 than Group 1 ($p<0.001$) and Group 2 ($p=0.001$). BOP percentage was significantly lower at Group 1 than Group 2 ($p=0.007$) and Group 3 ($p=0.004$). Molecular forms and relative levels of hBD-2 and hBD-3 in gingival tissue samples are given in Figure 1. Both hBDs were detected as dimers appearing at ~12 kDa. Additional 3 bands appeared at 25, 37, and 150 kDa and were defined as light and heavy chains of IgG, as previously described (Herrera et al., 2016). There were no significant differences at hBD-2 and hBD-3 levels between the groups (Figure 1).

In subgingival plaque samples, *P. gingivalis* counts and its gingipain activities, as determined with the fluorogenic protease substrate RR were significantly higher at sites with mild inflammation (group 2) and with moderate to severe inflammation (group 3) than at sites presenting solely with marginal inflammation (group 1) (Table 2). No difference was observed between the groups as regards to *P. gingivalis* counts and its gingipain enzyme activities in gingival tissue samples (Table 3).

All cytokine concentrations were above the lowest limit of detection (LLOD) except for IL-4 (29% under LLOD), IL-10 (22.9% under LLOD), IL-17F (16.6% under LLOD), and IL-23 (16.6% under LLOD). In descriptive analyses, cytokine concentrations below the minimum detection limit were substituted with a value of LLOD divided by 2 (Lubin et al., 2004). In gingival tissues with moderate to severe inflammation (group 3), IL-1β, IL-10, IL-17A, IL-17F, and IL-23 concentrations were higher than in tissues with mild inflammation (group 2) (Table 4).
There were negative correlations between gingival tissue protease activity and hBD-2 (p=0.033) and hBD-3 (p=0.003) (Table 5). IL-1 β (p=0.007), IL-4 (p=0.012), IL-17F (p=0.035), IL-23 (p=0.031), IL-25 (p=0.01), and IL-31 (p=0.039) showed positive and IL-33 (p=0.035) showed negative correlation with gingival tissue gingipain (RR) activity.

Discussion

The results revealed that hBD-2 and hBD-3 form dimers in gingival tissues and their levels negatively correlate with total protease activity. To the best of our knowledge, this study is the first to analyze hBD-2 and hBD-3 levels in gingival granulation tissue in relation to the hBD-suppressing pathogenic activity (including *P. gingivalis* count, and gingipain and total protease activities) and hBD-inducing inflammatory response (Th17-related inflammatory cytokines).
In the present study, the tissue samples were collected from periodontitis patients with various levels of clinical gingival inflammation, and granulation tissue of periodontal pocket was selected since it can be considered as the most active and appropriate site reflecting an ongoing disease. There was no ethical justification and clinical indication to excise sulcular tissue of healthy gingiva (no inflammation) from residual periodontal pockets with PPD 5-7 mm, thus no periodontally healthy control group was included in the protocol, which, however, may be regarded as a limitation of the study. Another limitation may be the fact that smokers were not included in the present study. The effect of smoking on tissue destruction in periodontal disease is well-described (Johnson and Guthmiller, 2007). Indeed, smoking may alter the innate immune response by suppressing hBD-2 in gingival epithelial cells as well (Mahanonda et al., 2009). A continuation study with smokers and quitters will enlighten the impact of smoking on the interactions between periodontal pathogens, inflammatory response, and gingival antimicrobial peptides. Finally, protease inhibitors were not used during sample storage and analysis in order not to interrupt the enzyme activity measurements. This may, at least partly, affect the levels of hBDs,

as they are prone to enzymatic degradation, since sample storage and preparation at 4 °C may not inhibit proteolysis totally.

hBDs are small cationic peptides of 4-5kDa. We found gingival hBD-2 and hBD-3 in dimer forms at around 12 kDa levels. Recently, dimeric forms of hBD-2 and hBD-3 at ~12 kDa were shown in human tonsil cells (Herrera et al., 2016). The authors demonstrated that additional three bands at 25, 37 and 150 kDa proved to be the light and heavy chains of IgG. These additional bands were also detected in gingival tissue (Figure 1). Our findings were validated by running the immunoblottings 1) at reduced conditions, 2) in the absence of primary antibodies, and 3) with different primary and secondary antibodies. In hBDs, dimerization is a precursor for higher oligomerization (Hoover et al., 2000). It was postulated that the presence of hBD-2 in the higher oligomeric forms facilitates its function by increasing cationicity and by enhancing the bacterial membrane permeability when compared with monomeric forms. In solutions, dominant oligomeric forms of hBD-2 have been found to be dimers (Hoover et al., 2000). Yet, it is unclear whether the dimerization observed in the present study is formed in the gingiva or after tissue extraction. Synthetic hBD-2 and hBD-3, which we used as positive controls, were also found in the same kDa region. As suggested by Suresh and Verma (2006), the observed peptide forms can be outcomes of the test conditions and/or high peptide concentrations. Thus, for confirming our findings, different extraction and analyzing techniques, (e.g., mass-spectrometry) will be needed.

According to our results, tissue levels of hBDs do not relate to the severity of gingival inflammation. Previous studies demonstrated controversial findings in terms of the relation between periodontal inflammation and hBD levels; RNA and protein expression profiles of hBDs in periodontitis and in experimental gingivitis models were found to be elevated, steady or suppressed (Offenbacher et al., 2009; Pereira et al., 2012; Yilmaz et al., 2015; Dommisch et al., 2015, 2019; Jourdain et al., 2019). Although our results are in line with the report of Li et al., (2016), in which no difference was observed in hBD-2 and hBD-3 gene expression levels between healthy and inflamed gingiva, longitudinal study designs are required to understand the shifts in inflammation-related regulation of hBD expression.

In the present study, elevated tissue levels of IL-1β, IL-10, IL-17A, IL-17F, and IL-23 were in line with the increase in inflammatory status of gingival samples. Previously, IL-1β and IL-17 have been shown to lead to an increased hBD-2 release from keratinocytes, while IL-10 decreased hBD-2 expression (Kanda et al., 2011). In our study, however, no relation was found between hBD levels and the amount of these inflammatory cytokines. On one hand, elevated inflammatory

cell (neutrophils, macrophages) infiltration to the sulcular granulation tissue may lead to a significant increase in cytokine levels. On the other hand, the disturbed epithelial structure and integrity in the granulation tissue may negatively affect the release of antimicrobial defensins from keratinocytes. Thus, characteristics of the sample tissue seem to have a major contribution to the levels of inflammatory cytokines and antimicrobial peptides.

Based on our results, a shift from marginal to mild inflammation is related to the elevated levels of *P. gingivalis* and related gingipain activity in plaque samples. On the other hand, no significant difference was found in *P. gingivalis* counts and gingipain activity between mild and severe inflammation groups. There are controversial results in regard to correlation between the levels of *P. gingivalis* and severity of gingival inflammation; both positive correlation (Demmer et al., 2008) and no correlation (Suda et al., 2004) have been presented. Indeed, it was also proposed that, as an opportunist bacterium, *P. gingivalis* can subvert host defense mechanisms to prolong its survival and establishment in the periodontal pocket, but at the same time does not aggravate the inflammatory response in order to disable adaptive immunity (Hajishengallis, 2011). It is possible to claim that the correlation between the degree of gingival inflammation and *P. gingivalis* counts and activity does not necessarily be linear, but may have exponential and stationary phases. However, the cross-sectional design of the present study does not allow us to differentiate these active interactions between the host response and bacterium.

To asses gingipain activity, two different fluorogenic substrates (RR and rR), with different amino acid configurations, were used. These two fluorogenic substrates have been developed to detect the presence of *P. gingivalis* by measuring gingipain activity with a high sensitivity and specificity (RR has a sensitivity of 70% and specificity of 96%, rR has a sensitivity of 95% and specificity of 73%) (Kaman et al., 2012). In our study, the tissue and plaque RR activities and plaque rR activity showed a positive correlation with the amount of *P. gingivalis* in the same sample. In addition, hBD-2 and hBD-3 levels had a negative correlation with total protease activity in gingival tissue, while no correlation was observed with gingipain activity. hBDs are known to be susceptible to degradation by bacterial proteases (Maisetta et al., 2011) and host proteases, such as by cathepsins (Taggart et al., 2003). Negative correlations between the total enzyme activity and hBD levels may indicate that the elevated bacteria- and host-induced protease activities in inflamed tissues may degrade or inactivate gingival tissue hBDs, leading to a shift from an insufficient innate immune response to a more aggressive humoral immune response.

To conclude, subgingival plaque levels of *P. gingivalis* and gingipain activity and tissue levels of Th17-related inflammatory cytokines, but not hBD levels in gingival tissue, are related to the severity of clinical periodontal inflammation. Negative correlations between the hBDs and gingival tissue total protease activity may indicate the degradation of these antimicrobial peptides in progressed inflammation, which was previously demonstrated only in in vitro studies.

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Table 1: Probing pocket depth (PPD), modified gingival index (MGI), clinical attachment level (CAL), plaque index (PI), bleeding on probing (BOP), and alveolar bone loss levels of each study group (Group 1: marginal inflammation, Group 2: mild inflammation, Group 3: moderate to severe inflammation).

	Group 1 n=13	**Group 2 n=16**	**Group 3 n=19**
PPD (mean ± st. dev.)	5.15 ± 0.56	5.38 ± 0.81	5.16 ± 0.38
% of sites with PPD 5 mm	92.3	81.2	84.2
% of sites with PPD 6 mm	0	0	15.8
% of sites with PPD 7 mm	7.7	18.8	0
MGI (mean ± st. dev.)	1 ± 0	2 ± 0	3.26 ± 0.45
CAL (mean ± st. dev.)	6.23 ± 1.17	6.31 ± 1.25	6.37 ± 0.9
% of sites with CAL >7 mm	84.6	81.25	84.2
% of sites with CAL 5-7 mm	15.4	18.75	15.8
PI (mean ± st. dev.)	1.38 ± 0.51	1.75 ± 0.45	2.37 ± 0.5
% of sites with BOP	61.5	100	100
% of sites with mild ABL	76.9	43.8	42.1
% of sites with moderate ABL	15.4	50	42.1
% of sites with severe ABL	7.7	6.2	15.8

Table 2: Subgingival plaque counts of *P. gingivalis* and its gingipain activities in the study groups (Group 1: marginal inflammation, Group 2: mild inflammation, Group 3: moderate to severe inflammation). Enzyme activities are presented in relative fluorescence per minute (RF/min). *p* values in bold indicate a significant difference ($p<0.05$) after multiple comparisons.

	Group 1 (n=13) median (min-max)	**Group 2 (n=16) median (min-max)**	**Group 3 (n=19) median (min-max)**	***p* value (Group 1 vs 2)**	***p* value (Group 1 vs 3)**	***p* value (Group 2 vs 3)**
P. gingivalis (count)	1643 (1 - 108012)	29529 (1 - 72257)	17468 (0 - 118284)	**0.012**	**0.001**	0.78
RR (RF/min)	0.85 (0.25 - 6.5)	5.1 (0.55 - 17.75)	5.65 (0.65 - 33.85)	**0.001**	**<0.001**	0.52
rR (RF/min)	0.35 (0 - 2.2)	0.78 (0 - 5.65)	0.55 (0 - 8.7)	0.056	0.14	0.66
PEK-054 (RF/min)	0 (0 - 35.75)	0.06 (0 – 53.88)	0.13 (0 - 180)	1	0.85	0.78

RR: Gingipain specific substrate that contains D-aminoacids; **rR:** Gingipain specific substrate that contains L-aminoacids; **PEK-054**: Substrate to determine total protease enzyme activity

Table 3: Gingival tissue counts of *P. gingivalis* and its gingipain activities in the study groups (Group 1: marginal inflammation, Group 2: mild inflammation, Group 3: moderate to severe inflammation). Enzyme activities are presented in relative fluorescence per minute (RF/min). *p* values in bold indicate a significant difference ($p<0.05$) after multiple comparisons.

	Group 1 (n=13) median (min-max)	**Group 2 (n=16) median (min-max)**	**Group 3 (n=19) median (min-max)**	***p* value (Group 1 vs 2)**	***p* value (Group 1 vs 3)**	***p* value (Group 2 vs 3)**
P. gingivalis **(**count/ng DNA**)**	6 (0 - 439)	11.5 (0 - 59)	4 (0 - 150)	0.81	0.71	0.35
RR (RF/min)	0.83 (0 – 9.67)	1 (0.5 – 37.2)	2.5 (0.17 – 65.5)	0.42	0.08	0.08
rR (RF/min)	46.3 (19.3 – 107)	55.08 (24.7 – 83.2)	35 (4.33 – 119)	0.95	0.38	0.32
PEK-054 (RF/min)	84.5 (35.2 – 128)	94.3 (38 – 130)	109 (16.2 – 172)	0.45	0.22	0.52

RR: Gingipain specific substrate that contains D-aminoacids; **rR:** Gingipain specific substrate that contains L-aminoacids; **PEK-054**: Substrate to determine total protease enzyme activity

Table 4: Tissue concentrations of examined cytokines (pg/ml) in the study groups (Group 1: marginal inflammation, Group 2: mild inflammation, Group 3: moderate to severe inflammation). *p* values in bold indicate a significant difference ($p<0.05$) after multiple comparisons.

	Group 1 (n=13) median (min-max)	**Group 2 (n=16) median (min-max)**	**Group 3 (n=19) median (min-max)**	***p* value (Group 1 vs 2)**	***p* value (Group 1 vs 3)**	***p* value (Group 2 vs 3)**
IL-1β	33.46 (8.21 –218)	37.31 (8.64 – 177)	95.78 (6.71 – 287)	0.861	0.088	**0.012**
IL-4	1.01 (0.26 – 7.87)	1.01 (0.26 – 3.88)	2.33 (0.26 – 5.51)	0.619	0.229	0.031
IL-6	8.64 (3.14 – 80.11)	10.38 (1.88 – 116)	36.81 (0.86 – 190)	0.693	0.13	0.037
IL-10	0.7 (0.15 – 3.64)	0.78 (0.15 – 3.4)	1.88 (0.15 – 7.18)	0.738	**0.049**	**0.024**
IL-17A	9.04 (2.26 – 30.94)	7.57 (3.44 – 15.55)	19.33 (4.56 – 39.44)	0.948	**0.037**	**0.002**
IL-17F	3.16 (0.4 – 16.21)	3.94 (0.4 – 9.21)	8.38 (0.4 – 23.75)	0.965	0.094	**0.006**
IL-21	22.43 (10.71 – 75.43)	17.8 (0.66 – 72.2)	36.03 (8.3 – 90.41)	0.301	0.818	0.191
IL-22	22.58 (12.85 – 60.21)	23.04 (10.97 – 36.74)	29.45 (9.07 – 53.01)	0.614	0.552	0.063

IL-23	12.16 (0.77 – 103)	12.16 (0.77 – 41.37)	17.29 (0.77 – 77.37)	0.548	0.326	**0.036**
IL-25	0.89 (0.29 – 7.87)	1.05 (0.34 – 2.65)	1.78 (0.34 – 5.24)	0.775	0.077	0.021
IL-31	76.5 (36.62 – 576)	80.45 (36.62 – 216)	147. 12 (28.54 – 344)	0.809	0.179	0.029
IL-33	146 (20.38 – 582)	101 (41.7 – 585)	56.92 (23.02 – 463)	1	0.242	0.04
IFN-γ	3.48 (0.71 – 14.61)	2.9 (0.71 – 29.19)	6.06 (0.5 – 27.97)	0.553	0.161	0.079
sCD40L	39.55 (16.99 – 96.48)	38.48 (12.21 – 61.83)	38.27 (13.18 – 111)	0.861	0.908	0.96
TNF-α	4.35 (1.5 – 24.95)	4.93 (1.42 – 23.83)	5.79 (0.82 – 16.03)	0.677	0.291	0.436

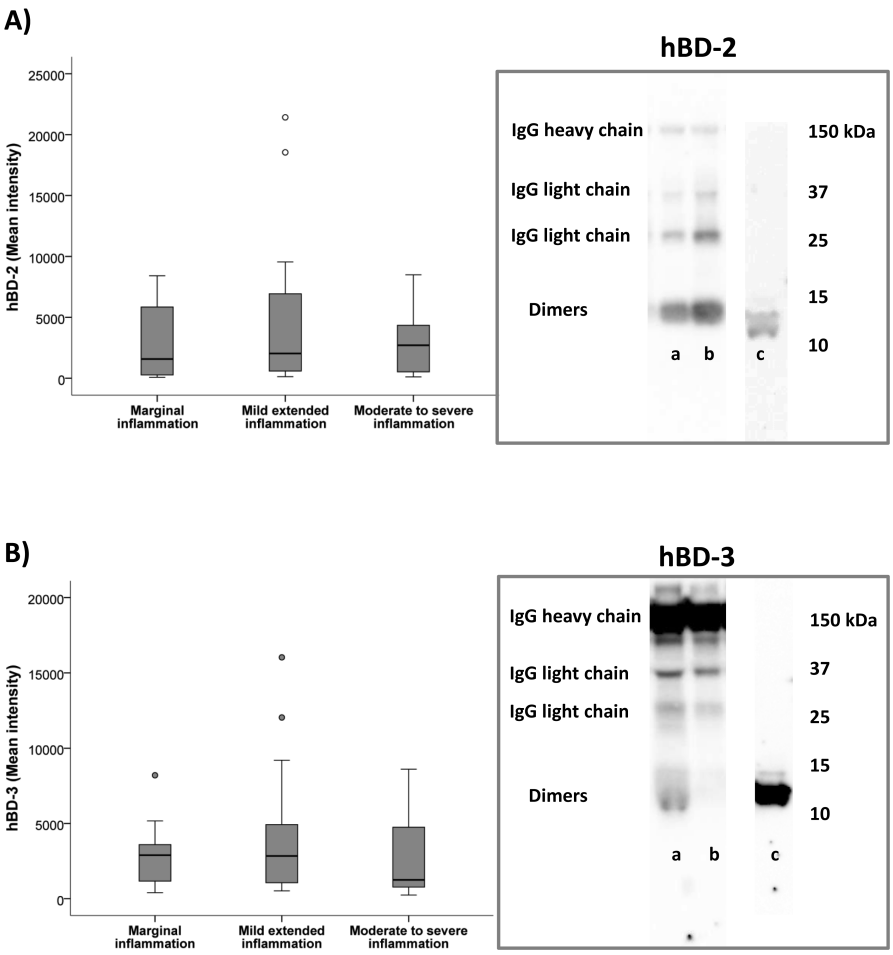
Table 5: Correlations between hBD-2, hBD-3, *P. gingivalis*, gingipain activity, and total protease activity levels. Statistically significant p values (p<0.05) are indicated with bold.

	P. gingivalis **plaque**	***P. gingivalis*** **tissue**	**hBD-2**	**hBD-3**
	Spearman correlation coefficient, *p*-value			
RR plaque	0.803, **<0.001**	0.198, 0.177	-0.078, 0.600	-0.010, 0.949
RR tissue	0.195, 0.177	0.383, **0.007**	0.167, 0.256	0.021, 0.889
rR plaque	0.401, **0.005**	0.334, **0.020**	-0.009, 0.950	0.076, 0.609
rR tissue	-0.142, 0.337	-0.237, 0.105	-0.041, 0.783	0.101, 0.496
PEK-054 plaque	-0.136, 0.357	-0.336, **0.019**	-0.146, 0.321	-0.092, 0.535
PEK-054 tissue	-0.035, 0.815	-0.308, **0.033**	-0.309, **0.033**	-0.417, **0.003**

RR: Gingipain specific substrate that contains D-aminoacids; **rR:** Gingipain specific substrate that contains L-aminoacids; **PEK-05**4: Substrate to determine total protease enzyme activity

Figure legends

Figure 1: Molecular forms and relative levels of hBD-2 (A) and hBD-3 (B) in human gingival samples with different degrees of inflammation. In the western-blot figures, lines a and b are gingival tissue samples, and c is a synthetic protein.



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